Structural organization of an encephalitic human isolate of *Banna virus* (genus *Seadornavirus*, family *Reoviridae*)

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*Banna virus* (BAV) is the type species of the genus *Seadornavirus* within the family *Reoviridae*. The Chinese BAV isolate (BAV-Ch), which causes encephalitis in humans, was shown to have a structural organization and particle morphology reminiscent of that of rotaviruses, with fibre proteins projecting from the surface of the particle. Intact BAV-Ch virus particles contain seven structural proteins, two of which (VP4 and VP9) form the outer coat. The inner (core) particles contain five additional proteins (VP1, VP2, VP3, VP8 and VP10) and are 'non-turreted', with a relatively smooth surface appearance. VP2 is the ‘$T=2$’ protein that forms the innermost ‘subcore’ layer, whilst VP8 is the ‘$T=13$’ protein forming the core-surface layer. Sequence comparisons indicate that BAV VP9 and VP10 are equivalent to the VP8* and VP5* domains, respectively, of rotavirus outer-coat protein VP4 (GenBank accession no. P12976). VP9 has also been shown to be responsible for virus attachment to the host-cell surface and may be involved in internalization. These similarities reveal a previously unreported genetic link between the genera *Rotavirus* and *Seadornavirus*, although the expression of BAV VP9 and VP10 from two separate genome segments, rather than by the proteolytic cleavage of a single gene product (as seen in rotavirus VP4), suggests a significant evolutionary jump between the members of these two genera.

INTRODUCTION

*Banna virus* (BAV) possesses a genome of 12 segments of double-stranded RNA (dsRNA) and was first isolated in 1987 from the cerebrospinal fluid (two isolates) and sera (25 isolates) of human patients with encephalitis (Xu et al., 1990; Chen & Tao, 1996) in Xishuang (Banna prefecture of Yunnan province, southern China). BAV has therefore been classified as a ‘biosafety level 3’ (BSL3) arboviral agent (http://www.cdc.gov/od/ohs/biosfty/bml3/bml4/bml4s74.htm). Additional strains of BAV were subsequently isolated in Xinjiang province (western China) from patients presenting milder clinical signs (fever and flu-like symptoms) (Xu et al., 1990; Li, 1992; Chen & Tao, 1996). These viruses are transmitted by *Anopheles* and *Culex* mosquitoes (Brown et al., 1993). Additional mosquito isolates of 12-segmented dsRNA viruses that are related antigenically to BAV have been reported from other provinces in China, including Beijing, Gansu, Hainan, Henan and Shanshi (Liting et al., 1995; Chen & Tao, 1996).

The BAV genome clearly identifies the virus as a member of the family *Reoviridae*, a large family of viruses containing 10, 11 or 12 segments of dsRNA, which currently includes a total of 12 distinct genera (Mertens, 2004; Mertens & Diprose, 2004; Mertens et al., 2004). The genomes of BAV and *Kadipiro virus* (KDV) have been sequenced (Attoui et al., 2000) and these data identify them as distinct species within the new genus *Seadornavirus* (type species BAV), family *Reoviridae* (Attoui et al., 2000, 2004a). However, until now, the morphology and biochemistry of the seadornaviruses have not been studied extensively.

Reoviruses (a term used here to indicate any member of the family *Reoviridae*) have been isolated from a wide range of
mammals, birds, reptiles, fish, crustaceans, marine protists, insects, ticks, arachnids, plants and fungi and include a total of 75 virus species, with ~30 further tentative species reported to date (Brussaard et al., 2004; Mertens et al., 2004). Reovirus particles have icosahedral symmetry with a diameter of approximately 60–85 nm. They are usually regarded as non-enveloped, although some can acquire a transient membrane envelope during morphogenesis or cell exit (Murphy et al., 1968; Martin et al., 1998; Mertens et al., 2000; Owens et al., 2004). The morphology of some reoviruses has been studied intensively by X-ray crystallography and cryo-electron microscopy (Prasad et al., 1988; Yeager et al., 1990, 1994; Grimes et al., 1998; Gouet et al., 1999; Hill et al., 1999; Reinisch et al., 2000; Diprose et al., 2001; Nason et al., 2004) and they can contain one, two or three concentric protein layers, identified here as 'core', 'core' and 'outer capsid', respectively. The inner-capsid layers and proteins are involved primarily in virus assembly and replication, and show a remarkable degree of structural conservation between different genera, exemplified by the subcore shell, which is constructed from 120 molecules of a single protein (Grimes et al., 1998; Reinisch et al., 2000; Mertens, 2004). In contrast, the outer-capsid proteins, which are involved in virus transmission, cell attachment and penetration, show greater variation, reflecting differences in the targeted host species, as well as responses to immune selective pressure by 'neutralizing' antibodies.

The reoviruses can be subdivided into two groups. The 'spiked' or 'turreted' viruses have 12 icosahedrally arranged projections (called turrets or spikes) situated on the surface of the icosahedral core particle, one at each of the fivefold axes (e.g. ortheo-reoviruses or cytopoviruses) (Baker et al., 1999; Hill et al., 1999; Nibert & Schiﬀ, 2001). In contrast, cores of the 'non-spiked' or 'non-turreted' viruses have a 'protein bilayer' structure, with a smooth or bristly surface appearance (e.g. rotaviruses or orbirviruses) (Grimes et al., 1998; Baker et al., 1999; Mertens et al., 2000, 2004).

We report a biomolecular study of the original Chinese strain of BAV (BAV-Ch), an isolate from the cerebrospinal fluid of a patient with encephalitis. Electron microscopy and electrophoretic analyses were used to identify the individual virus structural proteins and their location within the virus particle. The copy numbers of each protein present in purified virions were also determined, conﬁrming their individual structural and functional roles. Sequence analysis of the viral genome has been completed, helping to identify homologous proteins in other reoviruses, and a large subset of the viral proteins was expressed for antibody production, identifying two serotypes of BAV.

**METHODS**

**Virus propagation and purification.** BAV-Ch was propagated in *Aedes albopictus* C6/36 cells as described elsewhere (Attoui et al., 2000). Approximately 17–20 h post-infection (p.i.), the cells detach without lysis and assume a fusiform morphology. Extensive cell death is only observed after 4 days, reaching a maximum at 7 days p.i. At this point, the infected cells were pelleted (10 min at 2000 g, 4 °C) and the virus was concentrated from the supernatant by using 10% (w/v) PEG-8000, 2.5% (w/v) NaCl (overnight, 4 °C with stirring), followed by centrifugation (30 min, 5000 g, 4 °C). The pellet was suspended in 10 ml suspension buffer [150 mM NaCl, 250 mM sucrose, 1 mM MgCl₂, 4 mM CaCl₂, 10 mM Tris/HCl (pH 8.0)] and sonicated (three pulses of 150 W, 5 s each). BAV particles were purified by layering the suspension onto linear Percoll (Amersham Biosciences) gradients and centrifugation at 110 000 g (45 min, 10 °C). The light blue band containing virus particles was recovered, diluted in suspension buffer and pelleted at 150 000 g. A thin layer of colloidal silica forms at the bottom of the tube, which cushions the particles.

BAV cores were purified from the initial cell pellet. These were lysed in 5 ml deionized water (18 MΩ resistivity units) and run in a Potter homogenizer for 15 strokes, mixed vigorously with an equal volume of Vertrel-XF (Dupont) and centrifuged at 2000 g. The aqueous phase was layered onto either a discontinuous Optiprep (Sigma) gradient [10, 20, 30, 40 and 55% Optiprep in 100 mM Tris/HCl (pH 7.5)] or a caesium chloride gradient (Burroughs et al., 1994), then centrifuged at 10 °C for 2 h at 210 000 g. BAV particles were recovered at the interface of the 40 and 55% layers and subjected to a second round of caesium chloride ultracentrifugation. The resulting core particle band was harvested and centrifuged over a sucrose cushion [66% (w/w) in 100 mM Tris/HCl (pH 7.5), 10 mM MgCl₂] at 150 000 g for 2 h, then recovered and dialysed overnight at 25 °C against 100 mM Tris/HCl (pH 8.0), 10 mM MgCl₂.

**Electron microscopy.** Purified BAV particles were adsorbed onto Formvar/carbon-coated grids, stained with 2% potassium phosphotungstate or uranyl acetate and examined by using a Philips Morgagni 280 transmission electron microscope. Infected cells recovered at 24–30 h p.i. were pelleted by centrifugation at 600 g for 10 min and fixed for 1 h in 4% glutaraldehyde, post-fixed for 30 min in 1% phosphate-buffered osmium tetroxide, dehydrated in a graded ethanol series and embedded in an Araldite–Epon mixture (Mollenhauer, 1964). Thin sections were cut, stained with lead citrate (Rehse-Küpper et al., 1976) and examined by transmission electron microscopy (TEM).

**Sequence analysis of BAV-Ch genome segments 3, 4 and 5.** BAV-Ch dsRNA was extracted from concentrated purified virus by using RNA-Now reagent (Biogentex) and copied into cDNA as described previously (Attoui et al., 1998). The full lengths of segments 3, 4 and 5 were sequenced by using primers designed from segments 3, 4 and 5 of BAV-In6423 (GenBank accession nos AF134515–AF134517). The theoretical protein sequence was used for identiﬁcation of proteins by mass spectrometry (see below). The BAV-Ch sequences were compared with those of BAV-In6423 by using the BLAST program implemented in the DNATools package (version 5.2.018; S. W. Rasmussen, Valby Data Center, Denmark).

**Recombinant protein production and animal immunization.** Viral proteins VP7–VP12 of BAV-Ch and VP9 of BAV-In6969 were expressed as described previously (Mohd Jaafar et al., 2004). Briefly, segments 7–12 were cloned in vector pGEX-AT-2 to express glutathione S-transferase (GST)-fused proteins in *Escherichia coli* BL-21. Proteins were puriﬁed by glutathione affinity chromatography.

Mouse immune ascitic fluid (MIAF) against BAV-Ch virions was prepared by four initial intraperitoneal (IP) injections, at 2 week intervals, of 100 p.f.u. inactivated purified virus particles into 9-week-old mice. Similarly, MIAFs against individual recombinant-expressed proteins were also prepared for VP7–VP12 inclusive, using ~100 µg each protein. MIAF production was induced by injecting 0.5 ml pristane IP and ascitic fluid was recovered 12 days later.
Analysis of virus structural proteins by SDS-PAGE, mass spectrometry and Western immunoblotting. Percoll-purified whole virus was mixed with an equal volume of denaturation buffer [160 mM Tris/HCl (pH 6.8), 4 mM EDTA, 3.6% SDS, 60 mM dithiothreitol (DTT), 0.2% β-mercaptoethanol, 0.8% methionine] and heat-denatured at 95 °C for 3 min. The proteins were analysed by SDS-PAGE using 10% gels, then stained with 0.05% Coomassie blue in methanol/acetic acid/water (45:10:45).

Individual BAV proteins were identified by electrospray ionization mass spectrometry/mass spectrometry (Eurougec Proteomics). Protein bands were excised from gels and destained by using alternating high and low concentrations of acetonitrile/Tris/HCl (pH 8.1) [250 μl of a mixture of 250 mM Tris/HCl (pH 8.1) and 50% (v/v) acetonitrile (mg gel)^{-1}, followed by washing with 200 μl acetonitrile to dehydrate the gel]. DTT was added (65 mM final concentration) to reduce disulphide bonds, followed by alkylation of reduced cysteine residues by 2.5% (w/v) iodoacetamide. Bands were digested ‘in gel’ by using 44 μg porcine trypsin (mg gel)^{-1} (Roche) under non-reducing conditions in 50 mM Tris/HCl (pH 8.1) for 16 h at 35 °C. The resulting peptides were desalted by using ZipTips (Millipore) and analysed by using QStar XL (Applied Biosystems). MS data were acquired within the range of 100–2000 m/z. Peptides were also subjected to capillary liquid chromatography and separated on a C18 column using an acetonitrile/formic acid mixture (70% aqueous acetonitrile containing 0.1% formic acid) before injection into LCQ-FTICR and QTOF mass spectrometers. MS data were acquired within the range of 50–2000 m/z. The sequences of at least three peptides (10 aa or longer) were used to identify each protein derived from the purified particles by referring to the viral genome sequence.

The virus structural proteins separated by SDS-PAGE (whole virus purified on Percoll) were electroblotted onto nitrocellulose membranes. The presence of proteins VP7–VP12 was tested by Western immunoblotting with the corresponding ascitic fluids produced by the respective recombinant proteins. The cross-reactivity of VP9 from BAV-Ch and BAV-In6423 was tested by Western immunoblotting.

Radiolabelling and enumeration of the virion structural proteins. C6/36 cells grown in a 75 cm^2 flask were infected with BAV-Ch in Eagle’s minimum essential medium (EMEM). After incubation at 27 °C for 6 h under 5% CO2, the culture medium was replaced with methionine-deficient EMEM containing 50 μCi [35S]methionine ml^{-1}. Labelled cells were harvested and dissolved in denaturation buffer. Labelled proteins were analysed by SDS-PAGE as described above. The gel was dried and autoradiographed.

For enumeration of individual structural proteins from BAV-Ch particles, the cells were incubated in the presence of [35S]methionine for 3 days and virus was purified from the supernatant by centrifugation at 150 000 g for 1 h over 35% sucrose (w/v) in Tris/HCl (100 mM, pH 8.0). The proteins were separated by 10% SDS-PAGE using a malleable matrix (Protoprep; National Diagnostics). The gel was stained with Coomassie blue and individual protein bands were excised and melted as described by the manufacturer, then added to 5 ml Safe-Emulsifier scintillation fluid in polyethylene scintillation vials (Packard Instruments), mixed vigorously and counted in a Packard 460 liquid scintillation counter. Values for the number of proteins were calculated by using the ratio of each protein to the total protein content in the virus.

Phylogenetic relationships between seadornaviruses and rotaviruses. The sequences of the RNA-dependent RNA polymerases (RdRps) of different roviruses (see Supplementary Table in JGV Online) were used in phylogenetic analyses. The sequences were aligned by using CLUSTAL W (Thompson et al., 1994) and a tree was constructed by using MEGA2 (Kumar et al., 2001) with P-distance and Poisson correction. The alignment showed that the most conserved region among the polymerases lies within the core domain of the enzyme (located at similar positions in RdRps of different roviruses) between aa 697 and 835 of BAV. This region, and the whole of the polymerase sequence, were used in phylogenetic comparisons. The sequences of the other seadornavirus proteins were also compared with those of rotaviruses and other roviruses.

RESULTS

Completion of the BAV-Ch genome sequence analysis

The sequences of BAV-Ch genome segments 1, 2, 6, 7, 8, 9, 10, 11 and 12 have been reported previously (Attoui et al., 2000). BAV-Ch genome segments 3, 4 and 5 have now also been analysed (GenBank accession nos AY549307–AY549309), providing the complete sequence of the virus genome. Comparison with corresponding proteins of BAV-In6423 showed amino acid identity values of 88, 95 and 83% for VP3, VP4 and VP5, respectively.

Morphology and morphogenesis of virus particles

BAV-Ch virus particles purified from the supernatant of infected C6/36 cells were visualized by negative staining and electron microscopy (Fig. 1). The intact virion has a three-layered capsid structure, organized as two concentric capsid shells (‘core’ and ‘outer capsid’). The morphology and mean external diameter (72–75 nm) of the intact BAV-Ch virions stained with phosphotungstic acid (Fig. 1a) are typical of non-turreted roviruses (Mertens et al., 2004), having an appearance reminiscent of the rotaviruses, with fibre proteins projecting from the surface (Estes, 2001). However, the fibres in BAV are much more numerous and appear to be less extended than in rotaviruses. Similar structures were also observed on negatively stained KDV virions (Attoui et al., 2004a). Some of the unpurified BAV-Ch virus particles pelleted from infected tissue-culture supernatant had an envelope-like structure (data not shown), which may have been generated by budding through the cell membrane, as described for the orbiviruses (Martin et al., 1998; Owens et al., 2004).

In contrast, BAV particles purified by using CsCl or ‘Optiprep’ gradients have lost the outer-capsid layer and have a mean diameter of 52–55 nm (typical of rovirus cores; Baker et al., 1999; Mertens et al., 2000). The core ‘shell’ is formed as a protein bilayer (visible after staining with uranyl acetate; Fig. 1b) that is typical of the ‘nonturreted’ roviruses. The inner and outer layers are identified as ‘subcore’ and ‘core surface’, respectively. BAV cores do not have surface spikes or turrets, although ring-shaped capsomeres similar to those of orbivirus cores and rotavirus double-layered particles (Grimes et al., 1998; Estes, 2001) were visible (Fig. 1c).

Thin sections showed large, electron-dense structures within the cytoplasm of BAV-Ch-infected cells (Fig. 1d),
which correspond to the viral inclusion bodies (VIB) that are thought to be the main site of replication and particle assembly of other reoviruses (Brookes et al., 1993; Estes, 2001; Nibert & Schiff, 2001; Mertens & Diprose, 2004). Particles (~50 nm in diameter) with a smooth surface were detected mainly at the periphery of the VIB, although some particles were also observed within the VIB matrix. Virus particles were also detected within large vacuoles that were dispersed throughout the cytoplasm of the infected cell. These vacuoles contained multiple double-layered vesicles, lined with viral particles (~50 nm in diameter) at their inner surface (Fig. 1d); it is possible that this reflects some involvement of cellular membrane structures or organelles in virus morphogenesis, transport or replication (as reported previously for the rotaviruses; Jourdan et al., 1997; Sapin et al., 2002). Virus entry into cells by endocytosis [shown in a higher magnification (D1A)]; D2, viral inclusion body (VIB); D3, virions formed around the VIB; D4, vacuole structures containing virions (shown at a higher magnification in D4A; virions are indicated by a black arrow); D4B, cross section in the double-layered vesicles (white circle); D5, virus budding from the periphery of the cell [shown at a higher magnification (D5A)].

**Identification of structural and non-structural proteins**

Host-cell protein synthesis is shut off 2 h post-BAV infection of C6/36 cells (data not shown), and the shut-off is complete by 6 h p.i. The incorporation of [35S]methionine added to C6/36 cell cultures at 6 h p.i. was incorporated almost exclusively into 12 protein bands (resolved by SDS-PAGE; Fig. 2) that are thought to represent the different viral proteins (one protein per genome segment). Most of these have apparent molecular masses that agree with the theoretical sizes predicted by sequence analysis of the viral genome. The only exception is VP7, which migrates more slowly than expected.

Purified BAV-Ch virus particles contain seven structural proteins, each of which co-migrated with one of the radiolabelled proteins from infected cells (VP1, VP2, VP3, VP4, VP8, VP9 and VP10; Figs 2 and 3a). Only five of these proteins were also detected in cores (Fig. 3a), indicating that the outer coat (like those of the non-turreted orbiviruses and rotaviruses) is composed of two proteins (VP4 and VP9). Analyses of BAV-Ch structural protein sequences by mass spectrometry confirmed the identity of the core (VP1, VP2, VP3, VP8 and VP10) and outer capsid (VP9 and VP4) components (Table 1), demonstrating that VP5, VP6, VP7, VP11 and VP12 are non-structural proteins.

Proteins VP7–VP12 (inclusive) of BAV-Ch and VP9 of BAV-In6969 were expressed in *E. coli* as C-terminal GST fusion proteins and used for production of MIAFs. Antibodies generated against VP8 and VP10 reacted with...
homologous recombinant proteins and with VP8 or VP10 from BAV-Ch cores (by Western immunoblotting); antibodies against recombinant VP9 also reacted with VP9 from the outer-capsid layer (Fig. 4). VP9 appeared to be the most immunoreactive BAV protein. However, native and recombinant VP9 proteins of BAV-Ch (genotype A) failed to cross-react with anti-VP9 of BAV-In6969 (genotype B) and vice versa, indicating that VP9 is both antigenically variable and virus ‘type’-specific. This identifies BAV-Ch and BAV-In6969 as prototypes of two distinct BAV serotypes (A and B, respectively).

In contrast, although ascitic fluids generated against VP7, VP11 and VP12 also detected homologous viral proteins in infected cell lysates, they showed no reaction with components of purified particles, confirming that these proteins are non-structural.

**Enumeration of BAV structural proteins**

[^35S]Methionine-labelled BAV-Ch particles were purified and analysed by SDS-PAGE and the ratios of the different structural proteins were calculated (see Methods). VP2 and VP8 are the two most abundant proteins of the BAV core. The lower relative abundance and higher molecular mass of VP2 identifies it as the subcore-shell protein (equivalent to VP3 of Bluetongue virus (BTV) and VP2 of rotavirus; Mertens et al., 2000; Estes, 2001). In contrast, VP8 is smaller and more abundant, identifying it as the core-surface ‘T13’ protein. VP8 and VP2 have a molar ratio of 6:5 in purified BAV-Ch particles, identical to the ratio of 780/120 that was previously detected between the subcore and core-surface proteins of both BTV and rotavirus (Lawton et al., 1997; Grimes et al., 1998; Stuart et al., 1998; Mertens et al., 2000). On this basis, the numbers of the VP8 and VP2 molecules in the BAV core are assumed to be 780 and 120, respectively, allowing the mean copy number of the other protein components of virus particles or cores to be calculated (Table 2).

**Relationship between the seadornaviruses and rotaviruses**

It has previously been suggested that amino acid identity levels of ≥20% between individual proteins of different

![Fig. 2. Coding assignments of the dsRNA segments of the BAV-Ch genome: SDS-PAGE analysis of radiolabelled intracellular viral proteins. (a) Genome of BAV-Ch separated by 10% SDS-PAGE (segments designated S1–S12; Attoui et al., 2000); (b) intracellular radiolabelled proteins of BAV-Ch (non-structural proteins are identified as NS). Proteins identified by Attoui et al. (1998) are indicated by an asterisk.](http://vir.sgmjournals.org/1151)

<table>
<thead>
<tr>
<th>Protein nomenclature</th>
<th>Theoretical size (kDa)</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1 (pol)</td>
<td>138</td>
<td>Structural/core RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>VP2</td>
<td>108</td>
<td>Structural/core Inner-layer coat protein</td>
</tr>
<tr>
<td>VP3</td>
<td>82</td>
<td>Structural/core Capping enzyme</td>
</tr>
<tr>
<td>VP4</td>
<td>63</td>
<td>Structural/outer coat Outer-coat protein</td>
</tr>
<tr>
<td>VP4-NS</td>
<td>55</td>
<td>Non-structural Unknown</td>
</tr>
<tr>
<td>VP6-NS</td>
<td>48</td>
<td>Non-structural Unknown</td>
</tr>
</tbody>
</table>
| VP7-NS               | 35                     | Non-structural Protein kinase |*
| VP8                  | 33                     | Structural/core Outer-layer core protein |
| VP9                  | 30.5                   | Structural/outer coat Outer-coat attachment protein |
| VP10                 | 28.5                   | Structural/core Unknown |
| VP12-NS              | 24                     | Non-structural dsRNA-binding protein |*
| VP11-NS              | 20.5                   | Non-structural Unknown |

[^35S]Methionine-labelled BAV-Ch particles were purified and analysed by SDS-PAGE and the ratios of the different structural proteins were calculated (see Methods). VP2 and VP8 are the two most abundant proteins of the BAV core. The lower relative abundance and higher molecular mass of VP2 identifies it as the subcore-shell protein (equivalent to VP3 of Bluetongue virus (BTV) and VP2 of rotavirus; Mertens et al., 2000; Estes, 2001). In contrast, VP8 is smaller and more abundant, identifying it as the core-surface ‘T13’ protein. VP8 and VP2 have a molar ratio of 6:5 in purified BAV-Ch particles, identical to the ratio of 780/120 that was previously detected between the subcore and core-surface proteins of both BTV and rotavirus (Lawton et al., 1997; Grimes et al., 1998; Stuart et al., 1998; Mertens et al., 2000). On this basis, the numbers of the VP8 and VP2 molecules in the BAV core are assumed to be 780 and 120, respectively, allowing the mean copy number of the other protein components of virus particles or cores to be calculated (Table 2).

**Relationship between the seadornaviruses and rotaviruses**

It has previously been suggested that amino acid identity levels of ≥20% between individual proteins of different

![Fig. 3. SDS-PAGE of proteins of purified BAV-Ch and hypothetical representation of the organization of BAV-Ch particles. (a) Proteins contained in BAV-Ch whole virus; 2, proteins contained in the core particles of BAV-Ch; M, size marker labelled in kDa. (b) Non-stoichiometric schematic representation of a whole BAV-Ch particle, containing the five core proteins VP1, VP2, VP3, VP8 and VP10 and the two outer-coat proteins VP4 and VP9. This organization is suggested by structural data for BAV and the properties of other reoviruses.](http://vir.sgmjournals.org/1151)
reoviruses are significant, reflecting similar function and
common ancestry (Mertens et al., 2000, 2004). This sugges-
tion is based on a maximum amino acid identity of
15–20% between the RdRp sequences of viruses from
different genera [the only exception is the orthoreoviruses
and aquareoviruses, which have a significantly closer rela-
tionship (amino acid identity of ~20% between the RdRps
of BAV and KDV) and aquareoviruses, which have a signifi-
cantly closer relationship (amino acid identity of ~20% between
the RdRps of BAV and KDV)].

Comparisons of BAV VP9 (283 aa) with the outer-coat
proteins of other non-turreted reoviruses revealed size
and sequence similarities (~20% amino acid identity) to
the VP8* subunit (241 aa) of simian rotavirus A (strain
SA11) VP4 (GenBank accession no. P12976) (Fig. 5a).
Similarities (~26% amino acid identity) were also detected
between BAV VP10 and the VP5* subunit of simian SA11
rotavirus A VP4 (Fig. 5b), suggesting that VP9 and VP10
may have a collective role similar to that of rotavirus
outer-capsid protein VP4.

A local BLAST analysis also revealed that VP3 of BAV
has amino acid identity of 28% (between aa 467 and 540)
with the simian rotavirus group A capping enzyme VP3
(guanylyl- and methyltransferase) between aa 449 and
710. Interestingly, the VP3 of KDV (the other species of
seadornaviruses) has 21% amino acid identity over a
longer sequence (between aa 224 and 654) to the rotavirus
group A capping enzyme VP3 between aa 252 and 632.

The RdRps of rotaviruses and BAV or KDV have 17 and
19% amino acid identity, respectively, reflecting a similar
protein function, but confirming that these viruses belong
to two different genera of reoviruses (Mertens et al., 2000;
Attoui et al., 2004a, b). The phylogenetic tree constructed
by using the partial polymerase sequences (Fig. 6) shows
that rotaviruses and seadornaviruses are on adjacent
branches, indicating that they share a common, if distant,
phylogenetic origin. P-distance and Poisson correction
algorithms gave a tree with identical topology (similar to
the tree built from the complete polymerase sequences;
Attoui et al., 2000), in which four genera (Rotavirus,
Seadornavirus, Phytoreovirus and Orbivirus) are located
along the same evolutionary branch (although bootstrap

Table 1. Identification of structural proteins by mass spectrometry

Peptides were identified by mass spectrometry of gel-purified native structural proteins.

<table>
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<th>Protein (nomenclature/location)</th>
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<th>Molecular mass (kDa)</th>
<th>Peptide sequence</th>
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<tr>
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<td>Expected</td>
<td>Calculated</td>
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</tr>
<tr>
<td>VP4 (outer coat)</td>
<td>14</td>
<td>1538-64</td>
<td>1538-72</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1532-72</td>
<td>1532-80</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1854-90</td>
<td>1854-98</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>1911-95</td>
<td>1912-01</td>
</tr>
<tr>
<td>VP8 (T13/core)</td>
<td>12</td>
<td>1274-80</td>
<td>1274-72</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2034-20</td>
<td>2034-04</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>2344-19</td>
<td>2344-11</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>2785-79</td>
<td>2785-62</td>
</tr>
<tr>
<td>VP9 (outer coat)</td>
<td>10</td>
<td>1099-67</td>
<td>1099-62</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1236-72</td>
<td>1236-67</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1398-83</td>
<td>1398-77</td>
</tr>
<tr>
<td>VP10 (core)</td>
<td>13</td>
<td>1485-80</td>
<td>1485-83</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1690-00</td>
<td>1689-85</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2006-99</td>
<td>2007-09</td>
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<td></td>
<td>18</td>
<td>2150-00</td>
<td>2150-03</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2347-20</td>
<td>2347-10</td>
</tr>
</tbody>
</table>
values are low, as observed in all phylogenetic reconstructions of the family Reoviridae to date).

**DISCUSSION**

Until recently, detailed structural investigations of the reoviruses associated with human infections have focused on the orthoreoviruses, orbiviruses and rotaviruses, whilst the coltiviruses and seadornaviruses (despite their involvement in infections of the human central nervous system) have been largely ignored. We have recently reported data concerning the evolution and electron microscopy of the coltiviruses (Attoui et al., 2002a). We now describe the completed genome sequence, biochemical analyses and initial structural studies of BAV-Ch, an encephalitic human isolate of BAV, the type species of the genus Seadornavirus.

Electron microscopy, radiolabelling, SDS-PAGE, mass spectrometry, sequencing and serological comparisons of viral proteins were also used to analyse the virus-particle structure. These studies demonstrate that BAV is a non-turreted virus and has a double-layered core particle (with a smooth outline) that is typical of the non-turreted reoviruses [e.g. the orbiviruses – (Grimes et al., 1998)] and on the orthoreoviruses.

**Table 2.** Molar ratios and copy numbers of the structural proteins per purified whole BAV particle, calculated as described in the text

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molar ratio</th>
<th>Calculated copy number based on:</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP2</td>
<td>120 copies</td>
<td>1 VP2 (120 copies)</td>
</tr>
<tr>
<td>VP8</td>
<td>780 copies</td>
<td>1 VP8 (780 copies)</td>
</tr>
</tbody>
</table>

**Fig. 4.** Western immunoblot of BAV-Ch-infected C6/36 cells, shown as strips, using anti-recombinant VP7–VP12. M, Size markers labelled in kDa; A, tested with anti-VP7 antibodies; B, tested with anti-VP8 antibodies; C, tested with anti-VP9 antibodies; D, tested with anti-VP10 antibodies (faint bands detected with anti-VP10 at ~50 and ~70 kDa are indicated by asterisks); E, tested with anti-VP11 antibodies; F, tested with anti-VP12 antibodies; G, tested with anti-BAV-Ch antibodies.

**Fig. 5.** Alignments of VP8* of rotavirus with VP9 of BAV and VP5* of rotavirus with VP10 of BAV. (a) Alignment of rotavirus VP8* (residues 1–239 of the uncleaved rotavirus VP4) with BAV VP9; (b) alignment of rotavirus VP5* (residues 289–521 of the cleaved rotavirus VP5*) with BAV VP10. Both alignments were generated by CLUSTAL W. Identical residues are indicated by asterisks and similar residues are highlighted in grey. Amino acid identity, calculated with the help of the MEGA 2 software, was found to be 20% between rotavirus VP8 and BAV VP9 and 26% between rotavirus VP5* and BAV VP10.
rotaviruses (Estes, 2001). When viewed by negative staining and electron microscopy, intact BAV-Ch particles have an appearance that is similar to that of the rotaviruses, with ‘fibre’ proteins projecting from their surface. The mass distribution of BAV proteins in the core and outer coat is at a ratio of approximately 60:40%. This is comparable to the other non-turreted reoviruses, including rotavirus (57:43%) and the orbiviruses (54:46%). In contrast, the protein distribution of the turreted orthoreoviruses is 35:65%.

Thin-section TEM of BAV-infected cells showed intracellular virus factories (VI Bs), with virions concentrated at their periphery, that are similar to those generated by other reoviruses (Mertens et al., 2000). However, one distinctive feature of BAV replication is the presence of virions within double-layered vesicles, contained within larger vacuole structures that are dispersed throughout the cytoplasm of infected cells. These structures may be involved in transport of the virus particles to the cell membrane prior to release by budding, or could indicate some involvement of intracellular membranes in virus assembly, as reported previously for the rotaviruses (Jourdan et al., 1997; Sapin et al., 2002).

BAV particles were observed budding from the cell surface by thin-section electron microscopy. Considerable numbers of virus particles (approx. 40% of viral yield) were detected in the culture supernatant as early as 30 h p.i., although cells were not lysed until at least 4 days p.i. Virus particles surrounded by an envelope-like structure were found in material pelleted from the infected-culture supernatant. Orbiviruses can bud from the surface of persistently (non-lytically) infected insect and mammalian cells (Martin et al., 1998; Takamatsu et al., 2003; Owens et al., 2004), releasing membrane-enveloped virus particles. Budding of BAV particles without disrupting the cell-surface membrane may reflect its initial non-lytic multiplication in C6/36 cells.

BAV-Ch infection shuts off C6/36 cell protein synthesis, resulting in incorporation of $^{35}$Smethionine solely into viral proteins. The electrophoretic migration pattern and rate of polypeptides observed (one per genome segment) were in general agreement with genome sequence analyses. The only exception was VP7, which migrated slightly slower than expected.

Intact BAV-Ch virus particles contain seven structural proteins, five of which are situated in the virus core (VP1, VP2, VP3, VP8 and VP10). Based on their locations in the virus core, molecular masses and molar ratio, VP2 (120 copies) was identified as the putative BAV subcore-shell ‘T2’ protein and VP8 (780 copies) was postulated to be the core-surface ‘T13’ protein, allowing the numbers of other proteins in the BAV particle to be calculated. A BLAST sequence analysis identified BAV VP1 as the viral RNA polymerase, detecting conserved motifs also found in polymers of other reoviruses. These include SGEL at positions 714–717 [which conforms to the motif SG(E/K/L/R/S)(A/F/K/L/N/P/T)] and GDD at positions 759–761, which
is a core motif of the enzyme (Mertens et al., 2000). The BAV core is predicted to contain ~27 copies of VP1, which is twice that previously observed in the orthoreoviruses, orbiviruses and the rotavirus polymerases. This suggests packaging of two BAV polymerase molecules at each of the 12 icosahedral vertices.

BAV VP3 is the least abundant of the core structural proteins, with approximately seven copies detected per particle. The sequence of BAV and KDV VP3 is similar to that of VP3 of rotaviruses (the guanylyltransferase). BAV VP3 also exhibits guanylyltransferase activity (Mohd Jaafar et al., 2005a). Structural studies of other reoviruses suggest that transcriptase complexes may be situated at each of the fivefold axes of the core particle, possibly as one complex per genome segment (Payne & Mertens, 1983; Prasad et al., 1996; Grimes et al., 1998; Gouet et al., 1999; Zhang et al., 1999, 2003; Diprose et al., 2001; Pesavento et al., 2001). We would therefore have predicted at least 12 copies of VP3 per core particle, to allow capping of the nascent mRNAs synthesized at each transcription site.

Earlier sequencing studies demonstrated amino acid identities ranging from 72 to 100% between the proteins of different BAV isolates (Attoui et al., 2000). However, VP9 showed a maximum of 40% amino acid identity, identifying two distinct virus genotypes: genotype A, represented by the Chinese isolate, BAV-Ch, and genotype B, represented by several isolates from mosquitoes caught in Indonesia (Attoui et al., 2000). The outer coat of the BAV virion is composed of ~300 copies per particle of VP4 and VP9. The only other outer-coat protein from a reovirus that is known to be incorporated in similar numbers is VP5 of BTV, which exists as 360 copies (present as 12 trimers) per particle (Hewat et al., 1992; Schoenhn et al., 1997; Stuart et al., 1998; Mertens et al., 2000). Native and recombinant VP3 proteins of BAV-Ch (genotype A) and BAV-In6969 (genotype B) failed to cross-react by Western immunoblotting, indicating that VP9 is both antigenically variable and can be used to identify two serotypes, A and B (Mohd Jaafar et al., 2004).

The location of VP9 on the outermost capsid layer, together with sequence similarities to rotavirus VP8*, suggest an involvement in cell attachment and that it is a likely target for neutralizing antibodies. Indeed, the structure and function of VP9 have recently been analysed by X-ray crystallography (Mohd Jaafar et al., 2005b), demonstrating that it forms trimers and shows structural similarities to the VP8* subunit of rotavirus cell-attachment protein VP4. Recent findings suggest that at least the VP5* subunit of rotavirus VP4 could also form trimers (Dormitzer et al., 2004). Anti-VP9 antibodies (BAV-Ch VP9) were shown to neutralize virus infectivity, whilst soluble trimeric VP9 protein remarkably increased infectivity of BAV in C6/36 cells (Mohd Jaafar et al., 2005b). These findings identified the involvement of VP9 in cell attachment and penetration.

BAV VP10 (~260 copies per BAV core particle) appears to have no direct equivalent in the cores of other non-turreted reoviruses. However, sequence alignments showed significant sequence similarities with the VP5* subunit of simian rotavirus A outer-coat protein VP4 (Fig. 5). Antibodies to VP10 do not neutralize virus infection, although when used in immunoblotting of BVA-Ch proteins from infected cells, they identified a band of the expected size (~28 kDa), as well as two other bands at ~50 and ~70 kDa (Fig. 4d), indicating that VP10 may form dimers and trimers.

If BAV VP9 and VP10 have a collective role similar to that of the VP8*/VP5* subunits of rotavirus VP4 during the initiation of virus infection, it would suggest that VP10 may be present at the outer surface of the core and could form a ‘stalk-base’ for trimers of VP9 (outer-coat protein). It may be significant that these proteins are present in similar numbers per BAV particle (~300 and ~250, respectively). Rotavirus VP5* and VP8* are generated by proteolytic cleavage of VP4, increasing the specific infectivity of the rotavirus particle. The expression of BAV VP9 and VP10 from two separate genome segments also achieves separation of the two protein sequences, which may be functionally important. It suggests that a significant evolutionary jump has occurred between the members of the genera *Seadornavirus* and *Rotavirus*, which is reflected in the different numbers of genome segments (12 and 11, respectively).

BAV proteins VP5, VP6, VP7, VP11 and VP12 were detected in infected cells, but were not found in the purified virion and can therefore be regarded as non-structural. Sequence comparisons failed to identify roles for VP5, VP6 and VP11. Previously, VP7 was found to exhibit similarities to certain protein kinases (Attoui et al., 1998, 2000).

BAV and other seadornaviruses replicate efficiently in both mosquito and mammalian cell lines and are potential ‘emerging’ BSL3 pathogens that may pose a future threat to human health. In this study, we have completed the BAV-Ch genome sequence analysis and an initial characterization of the virus architecture and organization. A possible evolutionary link between the rotaviruses and seadornaviruses is proposed, based on similar virus-particle morphology and sequence similarities observed in the outer-coat, guanylyltransferase and polymerase genes and proteins between BAV and the rotaviruses.

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